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Specific Regulation of the Adaptor Protein Complex AP-3 by the Arf GAP AGAP1

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Summary

Arf1 regulates membrane trafficking at several membrane sites by interacting with at least seven different vesicle coat proteins. Here, we test the hypothesis that Arf1-dependent coats are independently regulated by specific interaction with Arf GAPs. We find that the Arf GAP AGAP1 directly associates with and colocalizes with AP-3, a coat protein complex involved in trafficking in the endosomal-lysosomal system. Binding is mediated by the PH domain of AGAP1 and the δ and σ3 subunits of AP-3. Overexpression of AGAP1 changes the cellular distribution of AP-3, and reduced expression of AGAP1 renders AP-3 resistant to brefeldin A. AGAP1 overexpression does not affect the distribution of other coat proteins, and AP-3 distribution is not affected by overexpression of other Arf GAPs. Cells overexpressing AGAP1 also exhibit increased LAMP1 trafficking via the plasma membrane. Taken together, these results support the hypothesis that AGAP1 directly and specifically regulates AP-3-dependent trafficking.

Introduction

ADP ribosylation factors (Arfs) are Ras-like guanine nucleotide binding proteins that mediate intracellular membrane trafficking. The six identified mammalian Arfs are divided into three classes. Class I includes Arf1, 2, and 3, class II includes Arf4 and 5, and class III includes Arf6 (Moss and Vaughan, 1998). Among the six Arf proteins, Arf1 and Arf6 are the best characterized. Each affects both membrane and actin remodeling at multiple intracellular sites (Randazzo et al., 2000b).

The effects of Arfs on membrane trafficking are mediated, at least in part, by membrane coat proteins including COPI (Rothman, 2002), the adaptor protein (AP) complexes 1, 3, and 4 (Boehm and Bonifacino, 2001; Le Borgne and Hoflack, 1998), and the Golgi-localized, γ-ear containing, Arf binding proteins (GGA) 1–3 (Robinson and Bonifacino, 2001). By binding to and consequently recruiting coat proteins to specific sites, Arf regulates trafficking from and to the Golgi apparatus, trans-Golgi network, and endosomes. AP-3 is an example of an Arf1-dependent clathrin adaptor that localizes primarily to endosomes. This complex is composed of four subunits named δ (~160 kDa), β3 (~140 kDa), μ3 (~47 kDa), and σ3 (~23 kDa) (Dell'Angelica et al., 1997a; Simpson et al., 1997). There are two isoforms of the μ, β, and σ subunits, named A and B. The ubiquitously expressed AP-3 contains μ3A and β3A subunits, whereas a brain-specific form contains μ3B and β3B subunits (Blumstein et al., 2001). σ3A and σ3B are both expressed ubiquitously. AP-3 participates in the trafficking of proteins to lysosome-related organelles including melanosomes and platelet dense granules (Kantheti et al., 1998; Dell'Angelica et al., 1999; Feng et al., 2002), and contributes to the production of synaptic vesicles (Faundez et al., 1998). In cells such as fibroblasts that do not have these specialized organelles, AP-3 plays a role in the sorting and transport of lysosomal membrane proteins to lysosomes (Le Borgne et al., 1998; Dell'Angelica et al., 1999).

The activity of Arfs is regulated by two groups of proteins, namely the guanine nucleotide exchange factors (GEFs; Jackson and Casanova, 2000) and the GTPase activating proteins (GAPs; Donaldson and Jackson, 2000). Fourteen Arf GEFs and 16 Arf GAPs have been described. The GAPs are categorized into three groups. Arf GAP1/3 belong to group 1 (Cukierman et al., 1995; Liu et al., 2001), while Git1/2 compose a second group (Premont et al., 1998; Vitale et al., 2000). The third group, the AZAPs, is structurally characterized by a core of PH, Arf GAP, and ANK repeat domains. Subgroups within the third group are distinguished by domains outside of the catalytic core. The prototype of this group, ASAP1 (Brown et al., 1998) and its closely related member PAPα (Andreev et al., 1999) contain SH3 and proline-rich domains. ACAPs contain coiled-coil domains (Jackson et al., 2000). ARAP1, 2, and 3 contain a Rho-GAP domain in addition to a sterile α motif (SAM) and multiple PH domains (Miura et al., 2002; Krugmann et al., 2002). A subgroup, known as AGAPs (Nie et al., 2002; Xia et al., 2003), has a GTP binding protein-like domain (GLD) and a split PH domain (Figure 1A).

Because a single Arf isoform functions at multiple sites, other proteins that interact with Arf must confer the specificity of Arf function. Given that the Arf GEFs and Arf GAPs outnumber the Arf proteins, a plausible hypothesis is that these proteins contribute to the site specificity of Arf action. Here, we report that AP-3 interacts with the Arf GAP AGAP1 both in vivo and in vitro. Exogenously expressed AGAP1 colocalizes with endogenous AP-3. Overexpression of AGAP1 disrupts the normal membrane association of AP-3, and trafficking to lysosomes is also affected. These data support the hypothesis that AGAP1 is a specific physiologic regulator of AP-3 on endosomes.

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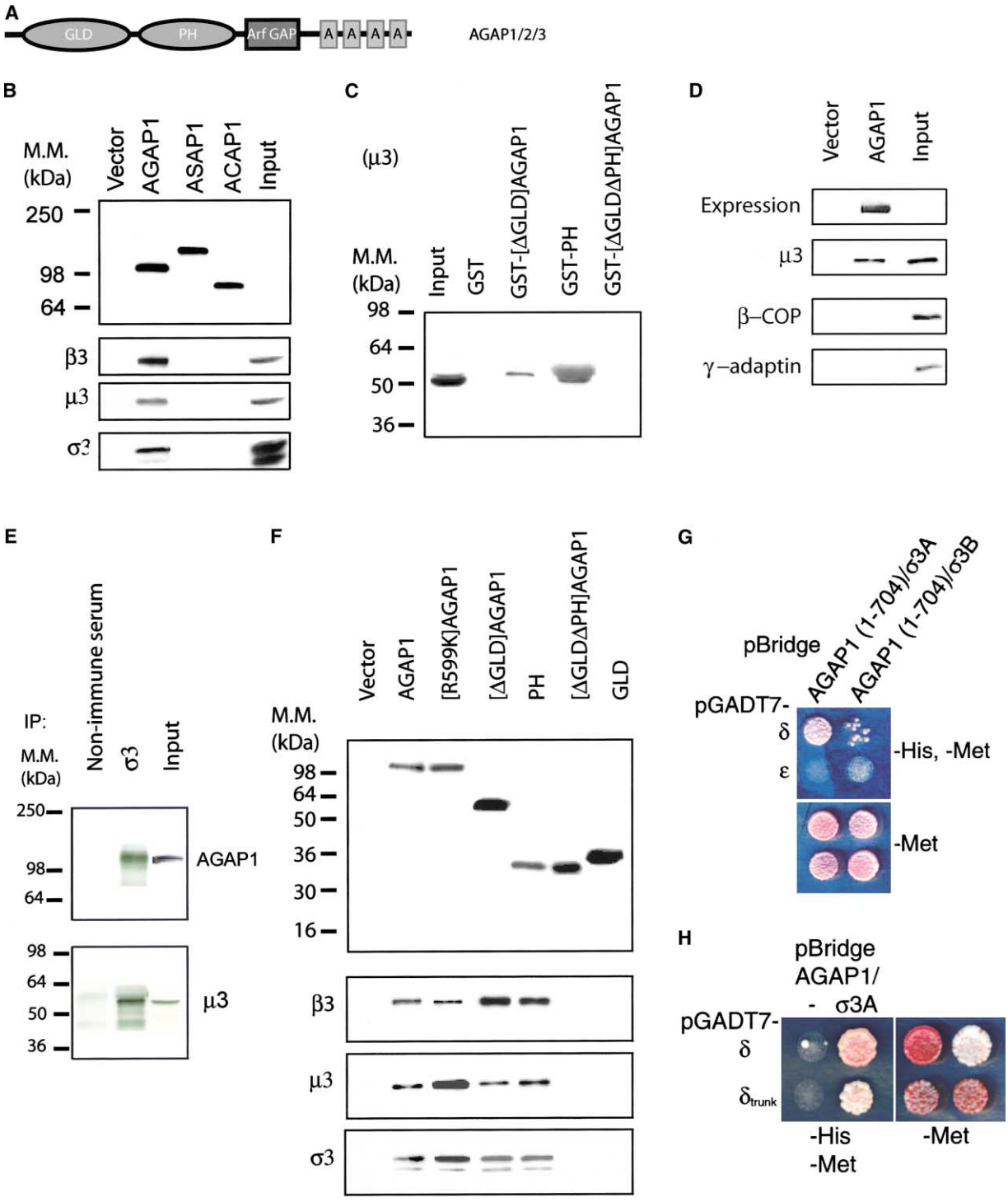

Figure 1. Specific Interaction between AGAP1 and AP-3

(A) Schematic of AGAP1. GLD, GTP binding protein-like domain; PH, plecksktrin homology; Arf GAP, Arf GTPase activating protein; A, ankyrin repeat (ANK).

(B) AP-3 coimmunoprecipitated with overexpressed AGAP1. NIH 3T3 cells were transfected with an empty vector or FLAG-tagged AGAP1, ASAP1, and ACAP1. The epitope-tagged proteins were immunoprecipitated with M2 antibody and eluted with FLAG peptide. The proteins were resolved on a Tris-glycine gel and the epitope-tagged proteins were detected by immunoblotting with M5 antibody (top panel). The coprecipitated AP-3 was detected with antibodies against the indicated subunits of AP-3 (bottom panels). The cell lysate was included as a positive control.

(C) In vitro binding between AP-3 and GST fusion proteins of AGAP1. Bovine brain cytosol was incubated with GST or GST-fused AGAP1 deletion mutants at 4°C overnight. Bound AP-3 was detected by immunoblot using an antibody against μ3. The bovine brain cytosol was included as a positive control.

Results

Specific Association of AGAP1 and AP-3

AGAP1 is an Arf1-specific Arf GAP involved in the endocytic pathway (Nie et al., 2002). To determine the specific site of AGAP1 action, we set out to identify proteins associated with AGAP1. One approach we used was to examine immunoprecipitates of AGAP1 for coprecipitated proteins. To this end, NIH 3T3 cells were transiently transfected with FLAG-tagged AGAP1. Proteins from cell lysates were immunoprecipitated with M2 anti-FLAG gel and eluted with FLAG peptide. Silver staining of gel-resolved proteins showed a band of ~140 kDa that coprecipitated with AGAP1. The protein was recovered and digested with trypsin. Tandem mass spectrometric analysis of the tryptic peptides revealed the identity of the associated protein as the β3A subunit of AP-3 (see Supplemental Data at http://www.developmentalcell.com/cgi/content/full/5/3/513/DC1).

Immunoblotting was used to confirm the identity of the β3A subunit and to detect the presence of two other subunits, μ3A and σ3 (A and B isoforms), of the AP-3 complex in immunoprecipitates (Figure 1B). A GST-[ΔGLD]AGAP1 fusion protein, expressed and purified from bacteria, was incubated with a soluble lysate from bovine brain. The fusion protein was precipitated with glutathione beads. As shown in Figure 1C, AP-3 was also present in the precipitates, consistent with formation of the complex in vitro.

AGAP1 discriminated among coat proteins regulated by Arf1. Epitope-tagged AGAP1 was immunoprecipitated from NIH 3T3 fibroblast and coimmunoprecipitated proteins were detected by immunoblotting. The signal for the μ3 subunit of AP-3 was equivalent to that for 10% of the cell lysate volume used for the immunoprecipitation. In contrast, neither β-COP, a subunit of COPI, nor γ-adaptin, a subunit of AP-1, was detected in the precipitate, although they were detected with similar signal intensity as μ3 in the cell lysates (Figure 1D). Conversely, AP-3 discriminated among GAPs. ASAP1 and ACAP1, two Arf GAPs closely related to AGAP1, were exogenously expressed to a similar level as AGAP1 in NIH 3T3 cells and immunoprecipitated in the same manner as AGAP1. Whereas AP-3 was detected in the precipitates containing AGAP1, none was detected with either ASAP1 or ACAP1 (Figure 1B). Endogenous AP-3 and AGAP1 also associated with each other. AP-3 was immunoprecipitated from bovine brain cytosol using an antibody to σ3. AP-3 and AGAP1 were detected in the immunoprecipitates by immunoblotting (Figure 1E). AGAP1 was not detected in the precipitate lacking AP-3 (Figure 1E).

Structural and Functional Requirements for Interaction

We next determined which domains in AGAP1 mediated the interaction of AGAP1 with AP-3. AP-3 was found to associate with [R599K]AGAP1, a point mutant of AGAP1 lacking GAP activity, and wild-type AGAP1 to a similar extent (Figure 1F). Thus, binding of AGAP1 to AP-3 is independent of its GAP activity. Next, we compared a series of epitope-tagged deletion mutants. Proteins lacking the PH domain had no detectable interaction, whereas proteins containing the PH domain, including a protein consisting of the PH domain alone, bound AP-3 (Figure 1F). In in vitro binding assays, GST fusion proteins containing the PH domain bound AP-3, whereas GST-[ΔGLDΔPH]AGAP1 did not (Figure 1C). The results indicate the binding was mediated by the PH domain.

We analyzed the subunits of AP-3 involved in interactions with AGAP1 using yeast two-hybrid and three-hybrid systems. Initial two-hybrid assays failed to reveal an interaction of AGAP1 with any single subunit of AP-3 (data not shown). Three-hybrid assays, however, demonstrated an interaction of AGAP1 with a combination of δ and σ3A and, less strongly, δ and σ3B (Figure 1G). These two subunits are known to assemble with one another as part of the AP-3 complex (Peden et al., 2002). The ε subunit of AP-4 did not interact with AGAP1 (Figure 1G). Thus, interactions with AGAP1 require both δ and one of the σ3 isoforms. Like other large adaptins, the δ subunit of AP-3 comprises three domains denoted trunk, hinge, and ear (Boehm and Bonifacino, 2001). σ3 assembles with the δ trunk as part of the AP-3 core. AGAP1 was found to bind to a σ3A-δ trunk dimer (Figure 1H).

AGAP1 and AP-3 Colocalize in Punctate Structures

The results presented above indicate that AGAP1 and AP-3 interact in vivo. We next examined whether AGAP1 and AP-3 also colocalized in cells. Because no antibodies good for immunofluorescence of AGAP1 are available, we transfected cells with epitope-tagged AGAP1. Because high levels of protein expression might affect an AGAP1-dependent compartment, we expressed both

(D) AGAP1 specifically coimmunoprecipitated AP-3. NIH 3T3 cells were transfected with an empty vector or [FLAG]AGAP1. The immunoprecipitated proteins were eluted with FLAG peptide, and immunoblot was performed to detect the FLAG-tagged AGAP1, as well as AP-3 (μ3), COP I (β-COP), and AP-1 (γ-adaptin).

(E) Association of endogenous AGAP1 and AP-3. Bovine brain cytosol was centrifuged at 100,000 × g for 15 min at 4°C. One hundred and fifty microliters each was incubated with 10 μl nonimmune sera or anti-σ3 at 4°C overnight. The samples were then incubated with prewashed GammaBind beads for 1 hr at 4°C. The samples were washed three times and resuspended in SDS-PAGE sample buffer. Immunoprecipitated AP-3 and AGAP1 were detected by immunoblot using antibodies against AGAP1 (top) and μ3 subunit of AP-3 (bottom). Bovine brain cytosol was also included as a positive control.

(F) PH domain of AGAP1 binds to AP-3. NIH 3T3 cells were transfected with FLAG-tagged wild-type AGAP1, a point mutant [R599K]AGAP1, which lacks GAP activity, and the indicated deletion mutants. Immunoprecipitation and immunoblotting was performed as in (B).

(G) Interaction between AGAP1 and AP-3 subunits δ and σ3 in the yeast three-hybrid system. δ and ε adaptin were coexpressed with AGAP1 (1–704) and σ3A or σ3B adaptin, and the interaction was detected by growth on selective −His and −Met plates.

(H) AGAP1 interacted with full-length δ and the trunk region of δ subunit of AP-3 in the yeast three-hybrid system. This interaction was dependent on the coexpression of σ3A protein.

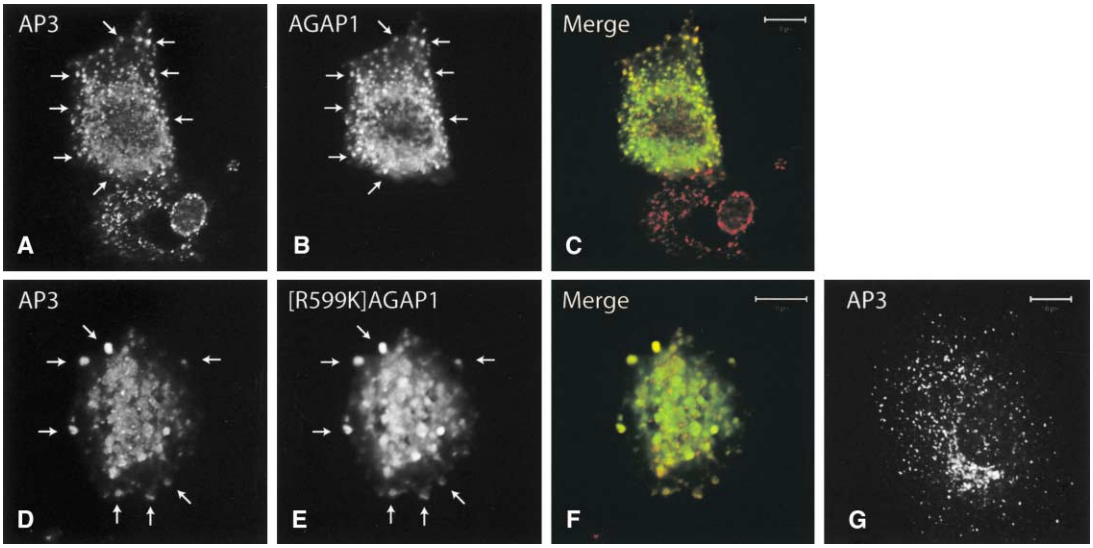

Figure 2. AGAP1 Colocalized with AP-3 in Punctate Structures in NIH 3T3 Cells

NIH 3T3 cells were transfected with FLAG-AGAP1 (A–C) or FLAG-[R599K]AGAP1 (D–F) in pSI vectors for 24 hr. Cells were replated on fibronectin-coated coverslips in Opti-MEM for 6 hr. The cells were fixed and stained for AP-3 (A and D), AGAP1 (B), or [R599K]AGAP1 (E). Colocalization with AP-3 was shown in the merged micrographs for AGAP1 (C) and [R599K]AGAP1 (F). Arrows indicate colocalized AP-3 and AGAP1 or [R599K]AGAP1. AP-3 staining in a nontransfected NIH 3T3 cell is shown in (G).

FLAG-AGAP1 and FLAG-[R599K]AGAP1 under the control of the SV40 promoter. In this system, the expression of both AGAP1 and [R599K]AGAP1 was at least 20 times lower than with the CMV promoter (data not shown). AP-3 staining appeared as punctate structures as described before (Figures 2A and 2G). Epitope-tagged AGAP1 also appeared in punctate structures, in many cases together with AP-3 (arrows in Figures 2A and 2B). Similarly, overexpressed FLAG-[R599K]AGAP1 colocalized with AP-3 in NIH 3T3 fibroblasts. However, the AP-3 compartment was altered by expression of the mutant, with AP-3 associated with larger punctate structures (Figure 2D), as compared to the nontransfected cells (Figure 2G) or wild-type AGAP1 transfected cells (Figure 2A). These results are consistent with AGAP1 associating with and regulating the AP-3 endosomes. Similar results were obtained using PC6 cells (not shown).

AGAP1 Regulates the Membrane Association of AP-3

The results with the [R599K] mutant of AGAP1 suggested a possible regulatory role in the AP-3 compartment. To further test for this regulatory role, we examined the effect of overexpressing AGAP1 to high levels on the cellular distribution of AP-3. To achieve high expression levels, we expressed AGAP1 under the control of the CMV promoter. As shown in Figure 2G, staining of AP-3 in untransfected NIH 3T3 cells appeared in punctate loci. When AGAP1 was overexpressed, AP-3 was associated with the punctate structures to a much lesser extent with some diffuse distribution (Figure 3A, panels a and b), suggesting dissociation of AP-3 from the membrane. This effect was specific for AGAP1. Several other members of the AZAP family Arf GAPs, ASAP1, ARAP1, ACAP1 (Figure 3A, panels c–h), and ACAP2 (not shown), did not affect the membrane association of endogenous AP-3 in NIH 3T3 cells. In addition, this effect of AGAP1 was specific for AP-3. While the membrane association of AP-3 was altered by AGAP1, the intracellular distribution of two other protein coats regulated by Arf1, COPI (as detected by β-COP; Figure 3B, panels a and b) and AP-1 (as detected by γ-adaptin; Figure 3B, panels c and d), was not changed by AGAP1 overexpression. Furthermore, this effect of AGAP1 on AP-3 was dependent on its GAP activity. The [R599K]AGAP1 mutant without GAP activity did not disrupt the membrane association of AP-3 (not shown). Therefore, inactivation of Arf1 by AGAP1 is required to dissociate AP-3.

Overexpression of AGAP1 led to AP-3 dissociation. If this effect was due to a specific physiologic interaction between AGAP1 and AP-3, then reducing AGAP1 levels should stabilize AP-3 on membranes. To test this prediction, we used siRNA to reduce AGAP1 expression (Figure 4A). In both control and siRNA-treated HeLa cells, AP-3 staining appeared as typical punctate structures widely distributed throughout the cytosol, with some degree of concentration in the perinuclear region (Figure 4B, panels a and b; Figure 4B, panels e and f). The cells were treated with brefeldin A (BFA) to disrupt the function of the Arf exchange factor. With GAP functioning normally, this treatment should reduce Arf-GTP in the AP-3 compartment, resulting in the dissociation of AP-3 from membranes. In cells with reduced GAP functioning in the AP-3 compartment, AP-3 should be relatively resistant to BFA. As predicted, in control cells, AP-3 dissociated from membranes after BFA treatment for 2 min (Figure 4B, panels c and d), whereas AP-3 remained associated with membranes after BFA treatment when AGAP1 expression was suppressed (Figure 4B, panels g and h). A reduction in AGAP1 did not affect

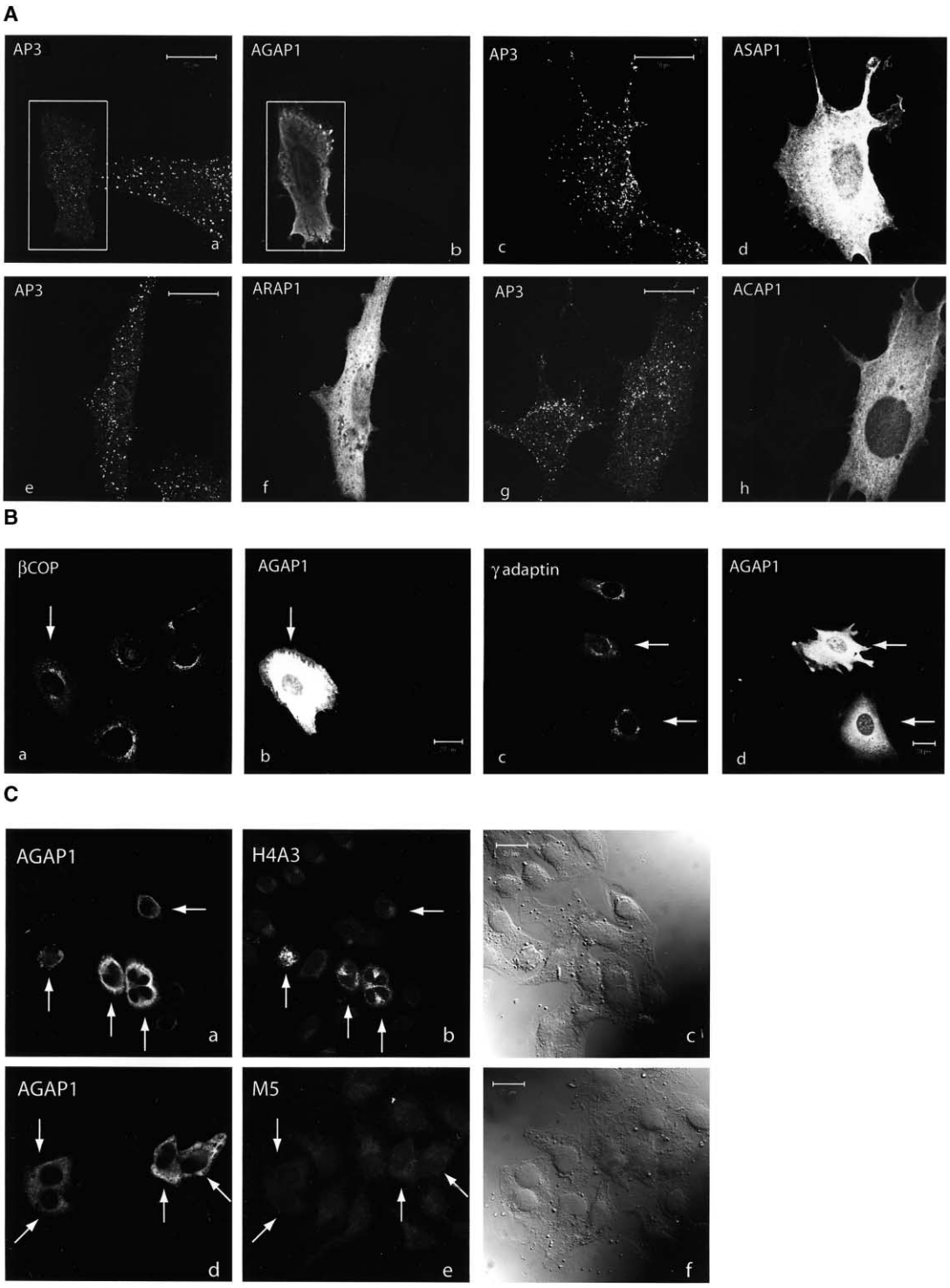

Figure 3. AGAP1 Overexpression Disrupted Membrane Association of AP-3 in NIH 3T3 Cells

(A) Panels a–h: AGAP1 disrupted AP-3 membrane association. NIH 3T3 cells were transfected with different Arf GAPs for 24 hr and the cells were replated on fibronectin-coated coverslips in Opti-MEM for 6 hr. The cells were then fixed and stained with antibodies against AP3 and the FLAG tag for Arf GAP.

Panels a and b: effect of AGAP1 on AP-3 distribution. The transfected cell is marked with the white box.

Panels c–j: no effect of other AZAP family members on AP-3 membrane association. Structurally related Arf GAPs such as ASAP1 (c and d), ARAP1 (e and f), and ACAP1 (g and h) did not change cellular distribution of AP-3 after overexpression.

(B) AGAP1 did not alter the cellular distribution of COPI and AP1. NIH 3T3 cells were transfected with [FLAG]AGAP1 for 24 hr and the cells were replated on fibronectin-coated coverslips in Opti-MEM for 6 hr. Cells were fixed and stained with antibodies against βCOP for COPI, and γ-adaptin for AP-1. The transfected cells were stained with antibody against the epitope tag and are marked with arrows.

(C) AGAP1 increased internalization of LAMP1 antibody in HeLa cells. HeLa cells were transfected with [FLAG]AGAP1 for 24 hr and the cells were replated on glass coverslips in DMEM containing 10% FBS for 6 hr. The monoclonal anti-LAMP1 (H4A3) or anti-FLAG (M5, as a negative control) was incubated with the cells at 0.5 μg/ml for 4 hr. The cells were then washed three times with DMEM containing 10% FBS, once with ice-cold PBS, and fixed. The transfected cells were stained with a polyclonal antibody against FLAG tag and are marked with arrows (panels a and d). The internalized antibodies were visualized using TRITC-conjugated goat anti-mouse secondary antibody (panels b and e). Phase contrast images were included to visualize nontransfected cells (panels c and f).

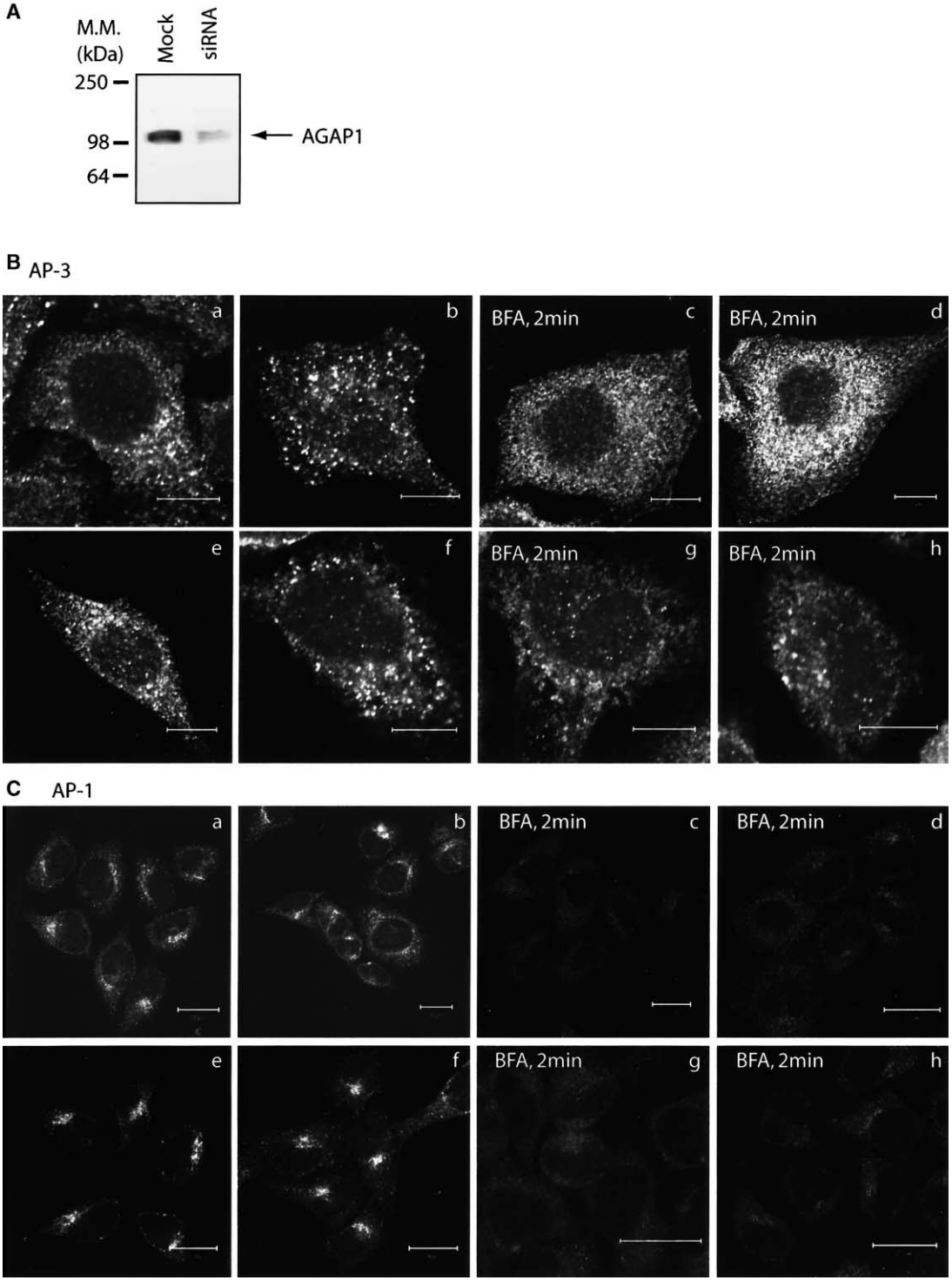

Figure 4. Inhibition of AGAP1 Gene Expression Conferred Resistance to BFA Treatment

(A) Immunoblot of AGAP1. HeLa cells were transfected with the siRNA without (Mock, lane 1) or with (siRNA, lane 2) oligofectamine for 3 days. The cells were harvested and cell lysates were subjected to SDS-PAGE. Endogenous AGAP1 was detected with a polyclonal antibody (indicated by the arrow).

(B) Staining of AP-3 in HeLa cells with mock transfection (upper panel) or siRNA transfection (lower panel). The cells were treated without (panels a, b, e, and f) and with (panels c, d, g, and h) BFA at 5 μg/ml for 2 min.

(C) Staining of AP-1 in HeLa cells with mock transfection (upper panel) or siRNA transfection (lower panel). Cells were treated without (panels a, b, e, and f) and with (panels c, d, g, and h) BFA at 5 μg/ml for 2 min.

BFA-induced dissociation of AP-1 from membranes (Figure 4C, panels g and h).

AGAP1 Altered AP-3-Dependent Trafficking of LAMP1

AP-3 regulates the traffic of lysosomal membrane proteins being transferred to lysosomes. If the pathway is disrupted, for instance by decreased AP-3 levels, lysosomal membrane proteins can still reach the lysosome but are rerouted through the plasma membrane. The altered pathway can be detected by incubation with antibodies against the lysosomal membrane proteins and measuring the internalization of the antibodies (Le Borgne et al., 1998; Dell'Angelica et al., 2000). We took this approach to determine whether AGAP1 affects AP-3-dependent trafficking. In HeLa cells, overexpression of AGAP1 increased the internalization of an antibody to LAMP1. The internalization of an irrelevant antibody, M5 monoclonal antibody, did not change (Figure 3C). These data are consistent with AGAP1 disrupting AP-3-dependent LAMP1 trafficking to lysosomes.

Discussion

Arf1 functions together with coat proteins to regulate membrane traffic at a number of different cellular sites. The mechanisms by which Arf1 can independently regulate the different coats at each site are not well defined. Accessory proteins that regulate the activation and inactivation of Arf have been proposed to have a role (Bonifacino and Jackson, 2003). Here, we provide the first example, to our knowledge, of an Arf GAP that regulates a specific endosomal compartment in mammalian cells. Furthermore, we show that site-specific activity of the GAP is dependent on direct binding to the coat being regulated.

The GAPs outnumber the Arfs. Localization studies indicate that the sites of action of various GAPs are probably different. Arf GAP1 is associated with the Golgi apparatus, and its overexpression affects Golgi structure (Huber et al., 1998), although it can also affect other structures when highly overexpressed (Ooi et al., 1998). ASAP1 associates with and affects the turnover of focal adhesions (Randazzo et al., 2000a). Other Arf GAPs associate with peripheral membranous structures including the plasma membrane (Turner et al., 2001; Matafora et al., 2001). To our knowledge, the association of a specific Arf GAP with a specific endosomal compartment has not been described prior to this study.

AGAP1 affects the function of AP-3 on endosomes, at least in part, by inactivation of Arf. As previously observed using dominant-negative Arf1, the ectopic expression of AGAP1 resulted in an altered cellular distribution of AP-3, with a loss of AP-3 associated with large ($\sim$0.5 μm) punctate structures that presumably represent the AP-3 endosomes. In addition, inhibition of AGAP1 gene expression by siRNA rendered AP-3 partly BFA resistant. The point mutant [R599K]AGAP1 did not induce the dissociation of AP-3 from the membrane although it was able to bind AP-3. On the contrary, AP-3 tended to be associated with larger size punctate structures when [R599K]AGAP1 was overexpressed. A plausible explanation is that [R599K]AGAP1, which lacks GAP activity, competes with endogenous AGAP1 for interaction with AP-3. By doing so, Arf1 function and AP-3 trafficking were less effective, leading to an accumulation of AP-3 in the putative AP-3 endosome.

The interaction between AGAP1 and AP-3 is analogous to the interaction between Arf GAP1 and coatomer in the COPI vesicle (Eugster et al., 2000). These results have led us to consider whether this mechanism of site-specific control of Arf generalizes to other coat proteins and GAPs. For instance, each member of the AZAP family of Arf GAPs may interact with a particular coat protein, such as AP-1, AP-3, and AP-4. In this way, the Arf GAPs might be integral components of the coats (Yang et al., 2002).

We are currently investigating whether the interaction of AP-3 with the PH domain of AGAP1 could regulate GAP activity. In ASAP1, another AZAP family member, the PH domain N-terminal to the GAP domain is a binding site for phosphoinositide 4,5-bisphosphate, an allosteric modulator of GAP activity (Kam et al., 2000). The PH domain of AGAP1 appears to function in an analogous manner, with phosphoinositide binding leading to stimulation of GAP activity. By interacting with the PH domain, AP-3 could either directly modify AGAP1 GAP activity or influence activation by phospholipids.

In summary, we have identified an interaction between AGAP1 and AP-3 that regulates AP-3 function on endosomes.

Experimental Procedures

Plasmids and Antibodies

The original cDNA of AGAP1 (KIAA1099) was obtained from Dr. Takahiro Nagase at the Kazusa DNA Research Institute. The constructs for FLAG epitope-tagged AGAP1 (amino acids 1–804), [R599K]AGAP1, [ΔGLD]AGAP1 (amino acids 347–804), [ΔGLD,ΔPH]AGAP1 (amino acids 556–804), PH (amino acids 347–535), or GLD (amino acids 69–317), as well as their corresponding GST fusion proteins, were obtained as previously described (Nie et al., 2002). The FLAG-AGAP1 and FLAG-[R599K]AGAP1 were also subcloned into the EcoRI and NotI sites of pSI (Promega). The monoclonal (mAb) and polyclonal anti-FLAG antibodies, as well as standard reagents, were from Sigma. The mAb against μ3, σ3, and γ-adaptin were from BD Biosciences. Polyclonal anti-β3 was described previously (Dell'Angelica et al., 1997b), and polyclonal anti-βCOP was from Affinity BioReagents. Monoclonal anti-LAMP1 (H4A3) was from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the Department of Biological Sciences, University of Iowa, Iowa City.

Cell Culture and Transfection

NIH 3T3 (clone 7; Willumsen et al., 1991), HeLa cells, and PC6 cells were cultured as described (Nie et al., 2002). Transfection was performed with Lipofectamine 2000 (Invitrogen) following the manufacturer's protocol. For the internalization of LAMP1 antibody, HeLa cells were incubated with H4A3 or M5 at 0.5 μg/ml for 4 hr. Cells were washed three times with DMEM containing 10% FBS, once with ice-cold PBS, and fixed with 2% formaldehyde.

GST Fusion Protein Pull-Down Assay

GST-fused AGAP1 and mutants were prepared as described (Nie et al., 2002). The fusion proteins were incubated with bovine brain cytosol in a total volume of 500 μl of 100 mM NaCl, 25 mM Tris HCl (pH 8.0), 1 mM DTT, and 0.1% Triton X-100 at 4°C overnight.

Immunoprecipitation

Cells were harvested into lysis buffer containing 25 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1% Triton X-100, 10% glycerol, and protease inhibitor cocktail (Roche). Cells were lysed with three freeze/thaw

cycles. The lysates were centrifuged at ~10,000 × g at 4°C for 10 min. The cleared lysates were incubated with M2 gel overnight. The samples were briefly centrifuged and the pellet was washed three times with the lysis buffer. The FLAG-tagged proteins were then eluted for 30 min with FLAG peptide at 4°C. For the immunoprecipitation of endogenous AP-3, bovine brain cytosol was incubated with antibody overnight at 4°C. Antibody was precipitated with GammaBind beads (Amersham Biosciences) as described by the manufacturer.

Immunofluorescence

Cells were prepared for immunofluorescence as described (Nie et al., 2002) and examined using a Zeiss Pascal confocal mounted on an Axioplan 2 microscope equipped with 63× and 100× Plan-Neofluar oil immersion lenses or a Zeiss LSM 510 confocal mounted on an Axiobert 100M microscope equipped with Plan-Apochromat 63×/1.4 oil DIC and Plan neofluar 100×/1.3 oil I immersion lens (Carl Zeiss).

Yeast Three-Hybrid System

The cDNAs for ϵ adaptin, full-length δ adaptin, or δ trunk (Boehm and Bonifacino, 2001) were subcloned into pGADT7. The cDNAs for AGAP1 amino acids 1–704 and σ3A or σ3B were subcloned into MCSI and MCSII of pBridge, respectively. Yeast HF7c were cotransformed with both plasmids according to the manufacturer's instructions (Clontech Matchmaker III manual), and cotransformants were selected on plates lacking leucine and tryptophan. Cotransformants were picked and selected for growth on plates lacking histidine, methionine, leucine, and tryptophan.

Preparation of siRNA and Transfection of HeLa Cells

Target-specific siRNA duplex with symmetric 3′ dTdT overhangs was synthesized at Dharmacon. The siRNA sequence targeting AGAP1 was from position 894–912 (5′-AGA AAG AGA UUG UCG UUG A-3′) of KIAA1099 (GenBank accession number AB029022). Transient transfection of HeLa cells with siRNAs was performed with Oligofectamine (Invitrogen) following the manufacturer's protocol. The cells were used 72 hr after transfection.

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